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Effects of Zn^{2+} chelators, DTPA and TPEN, and $ZnCl_2$ on the cells treated with hydrogen peroxide: a flow-cytometric study using rat thymocytes

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ABSTRACT

Recently, we have revealed that trace Zn^{2+} partly attenuates Ca^{2+}-dependent cell death induced by A23187, a calcium ionophore, in rat thymocytes. In this study, to see if Zn^{2+} attenuates the H_2O_2-induced cell death that is also Ca^{2+}-dependent, the effects of $ZnCl_2$ and chelators for Zn^{2+} have been examined by using a flow-cytometer with propidium iodide. The incubation with H_2O_2 increased the cell lethality. Simultaneous application of $ZnCl_2$ greatly augmented the H_2O_2-induced increase in lethality. DTPA, a chelator for extracellular Zn^{2+}, did not affect the increase in cell lethality by H_2O_2. However, the H_2O_2-induced increase in cell lethality was greatly attenuated by TPEN, a chelator for extracellular and intracellular Zn^{2+}. Taken together, it may be likely that intracellular Zn^{2+} modifies the H_2O_2-induced cytotoxicity. However, it cannot be ruled out the possibility that TPEN chelates intracellular Fe^{2+}, resulting in inhibiting the formation of hydroxyl radical from H_2O_2 that leads to an attenuation of H_2O_2 cytotoxicity.

Keywords: TPEN; DTPA; zinc; hydrogen peroxide; cytotoxicity

1. INTRODUCTION

Cell death induced by hydrogen peroxide (H_2O_2) in rat thymocytes is dependent on Ca^{2+} (Sakanashi *et al.*, 2008). Thus, the application of H_2O_2 increases intracellular Ca^{2+} concentration and the removal of external Ca^{2+} significantly attenuates the H_2O_2-induced cell death (Okazaki *et al.*, 1996; Nishizaki *et al.*, 2003; Sakanashi *et al.*, 2008). Sustained increase in intracellular Ca^{2+} concentration triggers either apoptotic or necrotic cell death (McConkey *et al.*, 1989; Azmi *et al.*, 1996; Berridge *et al.*, 1998; Orrenius *et al.*, 2003).

Recently, we have revealed that trace Zn^{2+} partly attenuates Ca^{2+}-dependent cell death induced by A23187, a calcium ionophore, in rat thymocytes (Sakanashi *et al.*, 2009). Zinc itself modifies or induces cell death in a concentration-dependent manner (MaCabe *et al.*, 1993; Jiang *et al.*, 1995; Iguchi *et al.*, 1998; Kolenko *et al.*, 2001; Truong-Tran *et al.*, 2001). Furthermore, zinc supplementation decreases oxidative stress induced by several types of compounds (DiSilvestro, 2000; Bao *et al.*, 2008; Szuster-Ciesielska *et al.*, 2009; Varghese *et al.*, 2009). Therefore, in this study, to see if Zn^{2+} attenuates the H_2O_2-induced cell death, the effects of $ZnCl_2$ and chelators for Zn^{2+}, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and diethylenetriamine-N,N,N',N",N"- pentaacetic acid (DTPA), on the cell death of rat thymocytes induced by H_2O_2 have been examined by using a flow-cytometer with propidium iodide.

Rat thymocytes were used for present study because of following reasons. First, the cell membranes of thymocytes remain intact because single cells can be prepared without enzymatic treatment. Second, the process of cell death is extensively studied in murine thymocytes (McConkey *et al.*, 1994; Tiso *et al.*, 1995; Rinner *et al.*, 1996; Winoto, 1997; Rennecke *et al.*, 2000; Quaglino and

Ronchetti, 2001; Ortiz *et al*., 2001; Thompson *et al*., 2003).

2. MATERIALS AND METHODS

2.1. Chemicals

H_2O_2 was purchased from Sumitomo Chemical Co. (Osaka, Japan). So-called chelators for Zn^{2+}, TPEN and DTPA, were obtained from Dojin Chemical Laboratory (Kumamoto, Japan). Propidium iodide was supplied from Molecular Probes Inc. (Eugene, Oregon, USA). Other chemicals (NaCl, $CaCl_2$, $MgCl_2$, KCl, glucose, HEPES, NaOH, and $ZnCl_2$) were also purchased from Woko Pure Chemicals.

2.2. Animals and cell preparation

This study was approved by the Committee for Animal Experiments in the University of Tokushima (No. 05279 for Y. Oyama).

The procedure to prepare cell suspension was similar to that previously reported (Chikahisa and Oyama, 1992; Chikahisa *et al*., 1996). In brief, thymus glands dissected from ether-anesthetized rats were sliced at a thickness of 400-500 μm with razor under an ice-cold condition (1-4ºC). The slices were triturated by gently shaking in chilled Tyrode's solution (in mM: NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, HEPES 5, with an appropriate amount of NaOH to adjust pH to 7.3-7.4) to dissociate thymocytes. Thereafter, the Tyrode's solution containing the cells was passed through a mesh (a diameter of 10 μm) to prepare the cell suspension (about 5×10^5 cells/ml). The beaker containing the cell suspension was water-bathed at 36ºC for 1 hr before the experiment.

2.3. Experimental protocol

H_2O_2 was added to cell suspension (2 ml cell suspension in 10 ml test tube). To examine the effects of $ZnCl_2$, DTPA, and TPEN, the agents were respectively added to the suspension just before applying H_2O_2. The cell density was about 5×10^5 cells/mL. The cells were incubated with respective agent and hydrogen peroxide at 36°C for 2 hr under room air condition. The data acquisition of fluorescence from 2×10^3 cells by a flow cytometer required 10 sec at least.

2.4. Fluorescence measurements of cellular and membrane parameters

The method for measurement of cellular and membrane parameters, including forward scatter and side scatter, using a flow cytometer equipped with an argon laser (CytoACE-150, JASCO, Tokyo, Japan) and fluorescent probe was similar to those previously described (Chikahisa and Oyama, 1992; Chikahisa *et al*., 1996). The fluorescence was analyzed by JASCO software (Ver.3XX, JASCO). As to chemicals used in this study, there was no fluorescence detected under our experimental condition.

To assess cell lethality, propidium iodide was added to cell suspension to achieve a final concentration of 5 μM. Since propidium stains dead cells, the measurement of propidium fluorescence from cells provides a clue to estimate the lethality. The fluorescence was measured at 2 min after the application of propidium iodide by a flow cytometer. Excitation wavelength for propidium was 488 nm and emission was detected at 600 ± 20 nm.

2.5. Statistics

Values were expressed as the mean ± standard deviation of 4 experiments. Statistical analysis was performed by using Tukey multivariate analysis. A P value of < 0.05 was considered significant.

3. RESULTS

3.1. Change of H_2O_2-induced increase in cell lethality of rat thymocytes by $ZnCl_2$, DTPA, or TPEN

There was no change in cell lethality of rat thymocytes after 2-3 h incubation with 10 μM $ZnCl_2$, 10 μM DTPA, 10 μM TPEN, or 0.1 % DMSO as a solvent for TPEN. The application of 10 mM H_2O_2 time-dependently increased the population of cells exerting propidium fluorescence, presumably dead cells, indicating an increase in cell lethality. The cell lethality at 2 h after the start of H_2O_2 application ranged from 24.6 % to 44.0 %. Since prolonged incubation of cells with 10 mM H_2O_2 further increased the cell lethality, the values of cell lethality did not reach a steady state. DMSO at 0.1 % did not affect the H_2O_2-induced increase in cell lethality in the case of 2 h incubation.

As shown in Fig. 1, the incubation with 10 mM H_2O_2 for 2 h increased the population of cells exerting propidium fluorescence. Simultaneous application of 10 μM $ZnCl_2$ greatly augmented the H_2O_2-induced increase in the population (Fig. 1). DTPA at 10 μM, probably chelating extracellular Zn^{2+}, did not affect the increase in cell lethality by 10 mM H_2O_2. However, the H_2O_2-induced increase in the population was greatly attenuated by 10 μM TPEN, probably chelating extracellular and intracellular Zn^{2+} (Fig. 1). It is noted that normal Tyrode's solution with rat thymocytes contains 200-300 nM Zn^{2+} derived from the cells (Sakanashi *et al*., 2009). Results are summarized in Fig. 2. $ZnCl_2$ significantly augmented the cytotoxicity of H_2O_2

while TPEN, but not DTPA, significantly attenuated it (Fig. 2). Thus, one may suggest that intracellular Zn^{2+} has an essential role in H_2O_2-induced increase in lethality.

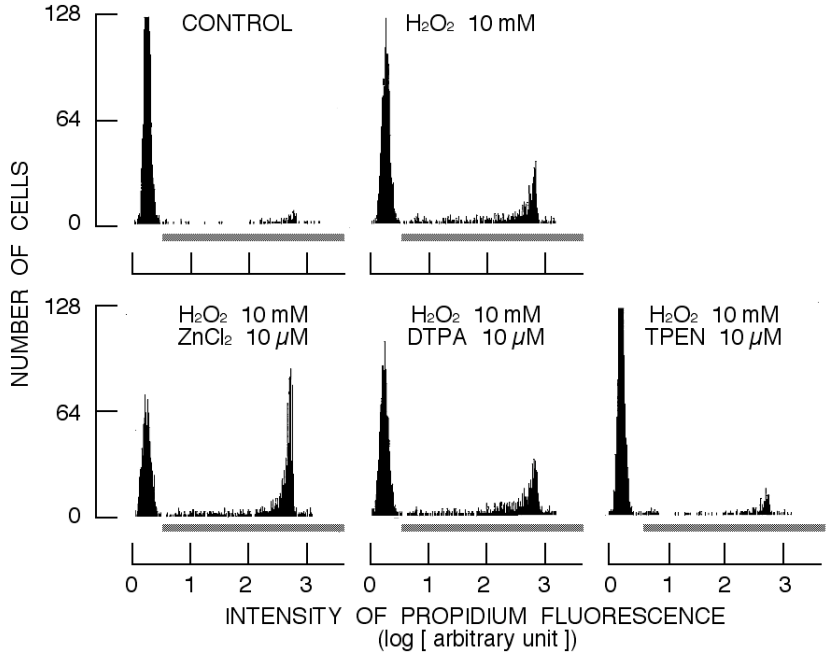

Fig.1. *Effects of $ZnCl_2$, DTPA, and TPEN on H_2O_2-induced increase in population of cells exerting propidium fluorescence. Each histogram was constructed from 2 □ 10^3 cells. Bar under the histogram indicates the region of cells exerting propidium fluorescence, dead cells.*

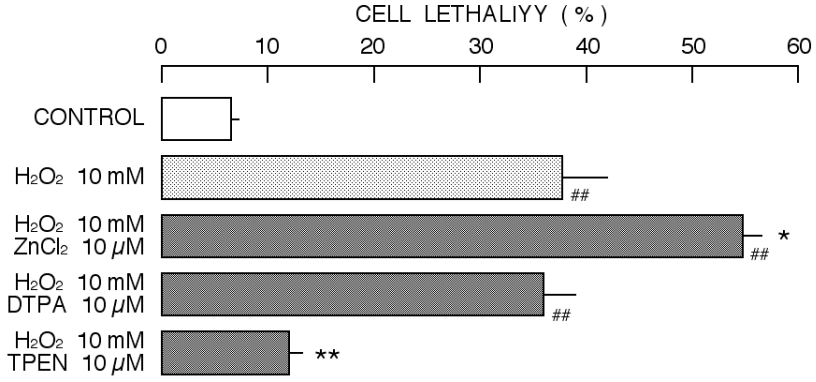

Fig. 2. *Cell lethality of H_2O_2-treated cells in simultaneous presence of $ZnCl_2$, DTPA, or TPEN. Column and bar respectively indicate mean and standard deviation of four experiments. Symbol (##) near column indicates significant change ($P < 0.01$) to control group (CONTROL). Asterisks (* and **) show significant difference ($P < 0.05$ and $P < 0.01$, respectively) to the group of cells treated with H_2O_2 alone. It is noted that the difference between control group and the group of cells incubated with H_2O_2 and TPEN.*

3.2. Change of H_2O_2-induced increase in population of shrunken cells by $ZnCl_2$, DTPA, or TPEN

Cytogram (forward scatter *versus* side scatter) was also affected by the incubation of cells with 10 mM H_2O_2 for 2 h. As shown in Fig. 3, the population of area S increased in the presence of H_2O_2. Simultaneous application of 10 mM H_2O_2 and 10 μM $ZnCl_2$ further increased the population of area S while 10 μM TPEN significantly attenuated the H_2O_2-induced increase in the population (Fig. 3). DTPA at 10 μM did not significantly affect the population changed by 10 mM H_2O_2. Results are summarized in Fig. 4. $ZnCl_2$ significantly augmented the H_2O_2-induced change in cytogram while TPEN significantly attenuated it. DTPA did not affect the H_2O_2-induced change. Therefore, it is likely that the cell shrinkage is well-associated with the increase in cell lethality.

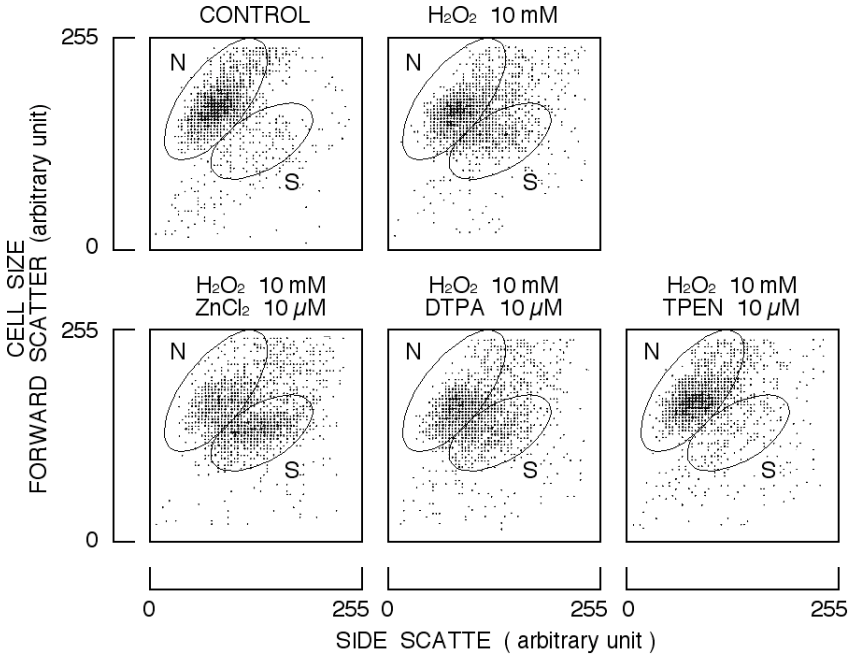

Fig. 3. *Effect of H_2O_2 on cytogram (forward scatter versus side scatter) of rat thymocytes in simultaneous application of $ZnCl_2$, DTPA, or TPEN. Each cytogram was constructed with 2 × 10^3 cells. Areas N and S indicate normal size cells and shrunken cells, respectively.*

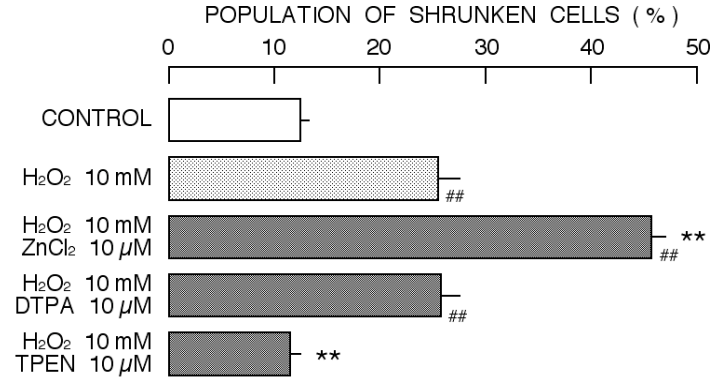

Fig. 4. *Proportion of shrunken cells in the cells treated with H_2O_2 in simultaneous presence of $ZnCl_2$, DTPA, or TPEN. Column and bar respectively indicate mean and standard deviation of four experiments. Symbol (##) near column indicates significant change ($P < 0.01$) to the control group (CONTROL). Asterisk (**) shows significant difference ($P < 0.01$) to the group of cells treated with H_2O_2 alone.*

4. DISCUSSION

DTPA is a chelator for extracellular Zn^{2+} while TPEN is one for both extracellular and intracellular Zn^{2+}. Therefore, it is supposed that chelating intracellular Zn^{2+} is essential in attenuation of H_2O_2-induced cytotoxicity. The application of $ZnCl_2$ increases intracellular Zn^{2+} concentration (Matsui *et al.*, 2008). Thus, $ZnCl_2$ may augment H_2O_2-induced cytotoxicity by increasing intracellular Zn^{2+} concentration. Therefore, it may be presumably suggested that intracellular Zn^{2+} augments H_2O_2-induced cytotoxicity. Although this suggestion well corresponds to the results in this study, it is challenged by previous findings. First, the cell death induced by H_2O_2 in rat thymocytes is significantly dependent on Ca^{2+} (Okazaki *et al.*, 1996; Nishizaki *et al.*, 2003; Sakanashi *et al.*, 2008). Second, TPEN potentiates the Ca^{2+}-dependent cytotoxicity of A23187 (Sakanashi *et al.*, 2009). Thus, it is also supposed that the application of TPEN augments the cytotoxicity of H_2O_2 because the H_2O_2 cytotoxicity is Ca^{2+}-dependent. However, the cytotoxicity of H_2O_2 is dependent on Fe^{2+}, rather than Ca^{2+} (Walker and Shah, 1991; Hiraishi *et al.*, 1993; Byler *et al.*, 1994; Lomonosova *et al.*, 1998). The combination of H_2O_2 and Fe^{2+} generates hydroxyl radical by Fenton reaction. Hydroxyl radical increases intracellular Ca^{2+} concentration (Dreher and Junod, 1995; Burlando and Viarengo, 2005). TPEN can chelate not only Zn^{2+} but also Fe^{2+}. Therefore, it is supposed that TPEN can suppress the generation of hydroxyl radicals by chelating intracellular Fe^{2+}, resulting in attenuation of H_2O_2-induced cytotoxicity. Thus, the role of intracellular Zn^{2+} in H_2O_2 cytotoxicity cannot be strongly addressed at present although TPEN is known to be a chelator for intracellular Zn^{2+}.

The H_2O_2-induced increase in cell lethality was augmented by adding $ZnCl_2$ (Figs. 1 and 2). In present study, we did not monitor the $ZnCl_2$-induced change in intracellular Zn^{2+} concentration during H_2O_2 exposure. However, the addition of $ZnCl_2$ may further elevate intracellular Zn^{2+} concentration since the application of H_2O_2 increases intracellular Zn^{2+} concentration (Hashimoto *et al.*, 2009). Excess increase in intracellular Zn^{2+} concentration leads to cell death (Kim *et al.*, 1999; Hamatake *et al.*, 2000; Iitaka *et al.*, 2001). In this aspect, further study will be necessary to elucidate the role of intracellular Zn^{2+} in the augmentation of H_2O_2 cytotoxicity.

As to the population of shrunken cells (Figs. 3 and 4), one of parameters during an early stage of apoptosis, TPEN almost completely suppressed the H_2O_2-induced increase in shrunken cell population while $ZnCl_2$ greatly increased the population. Cell shrinkage is associated with an activation of Ca^{2+}-dependen K^+ channels in rat thymocytes (Horimoto *et al.*, 2006). Therefore, in the case of simultaneous presence of TPEN, it is unlikely that H_2O_2 increases intracellular Ca^{2+} concentration, as suggested above. On the other hand, the combination of $ZnCl_2$ and H_2O_2 may further increase not only intracellular Zn^{2+} but also intracellular Ca^{2+} concentration. In this aspect, further studies on intracellular Ca^{2+} and Zn^{2+} concentrations will be required.

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